EASTERN REGIONAL RESEARCH CENTER
AGRICULTURAL RESEARCH SERVICE
UNITED STATES DEPARTMENT OF AGRICULTURE
600 E. MERMAID LANE
WYNDMOOR, PA 19038
(215) 233-6400

Title: Effects of Community Versus Single Strain Inoculants on the Biocontrol of *Salmonella* and Microbial Community Dynamics in Alfalfa Sprouts

Author(s): A. Matos, and J.L. Garland

Citation: Journal of Food Protection (2005) 68:(1) 40-48

Number: 7545

Effects of Community Versus Single Strain Inoculants on the Biocontrol of *Salmonella* and Microbial Community Dynamics in Alfalfa Sprouts†

ANABELLE MATOS[1]* AND JAY L. GARLAND[2]

[1]*U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, Pennsylvania 19038; and* [2]*Dynamac Corporation, Mail Code DYN-3, Kennedy Space Center, Florida 32899, USA*

MS 04-49: Received 9 February 2004/Accepted 19 July 2004

ABSTRACT

Potential biological control inoculants, *Pseudomonas fluorescens* 2-79 and microbial communities derived from market sprouts or laboratory-grown alfalfa sprouts, were introduced into alfalfa seeds with and without a *Salmonella* inoculum. We examined their ability to inhibit the growth of this foodborne pathogen and assess the relative effects of the inoculants on the alfalfa microbial community structure and function. Alfalfa seeds contaminated with a *Salmonella* cocktail were soaked for 2 h in bacterial suspensions from each inoculant tested. Inoculated alfalfa seeds were grown for 7 days and sampled during days 1, 3, and 7. At each sampling, alfalfa sprouts were sonicated for 7 min to recover microflora from the surface, and the resulting suspensions were diluted and plated on selective and nonselective media. Total bacterial counts were obtained using acridine orange staining, and the percentage culturability was calculated. Phenotypic potential of sprout-associated microbial communities inoculated with biocontrol treatments was assessed using community-level physiological profiles based on patterns of use of 95 separate carbon sources in Biolog plates. Community-level physiological profiles were also determined using oxygen-sensitive fluorophore in BD microtiter plates to examine functional patterns in these communities. No significant differences in total and mesophilic aerobe microbial cell density or microbial richness resulting from the introduction of inoculants on alfalfa seeds with and without *Salmonella* were observed. *P. fluorescens* 2-79 exhibited the greatest reduction in the growth of *Salmonella* early during alfalfa growth (4.22 log at day 1), while the market sprout inoculum had the reverse effect, resulting in a maximum log reduction (5.48) of *Salmonella* on day 7. Community-level physiological profiles analyses revealed that market sprout communities peaked higher and faster compared with the other inoculants tested. These results suggest that different modes of actions of single versus microbial consortia biocontrol treatments may be involved.

At least 21 food poisoning outbreaks due to the consumption of sprouts contaminated with *Salmonella* and *Escherichia coli* O157:H7 have occurred in the United States since 1995 *(6, 24)*. In 1999, the U.S. Department of Health and Human Services issued a consumer advisory warning of the risk associated with eating raw sprouts *(13)*. More recently, the U.S. Food and Drug Administration *(6)* updated its warning about the dangers of eating raw or lightly cooked sprouts to include mung bean sprouts as well as alfalfa sprouts ((*4*) and release of 19 August 2004 at www.cfsan.fda.gov/~dms/sproltr.html). The recommended safety step of soaking seeds in a calcium hypochlorite solution has not been proved to be completely effective *(5)*, indicating that alternative disinfection treatments, such as biological control, are needed.

The typical process of developing biological control treatments involves the initial isolation of potential biocontrol agents, followed by ecological, mode of action, and formulation studies leading to commercialization. A successful example of this approach is the use of fluorescent pseudomonads for the biological control of plant root pathogens *(2, 22)* because of their production of metabolites such as antibiotics *(19)* and siderophores *(17)* that inhibit life processes of bacteria and fungi.

An alternative biological control approach involves the use of defined mixtures of competitive exclusion cultures, rather than single strains, to limit the growth of pathogens. Defined competitive exclusion cultures, termed bacterial compositions, have been successfully used to prevent enteropathogen colonization in poultry and swine *(10, 15, 18)*. The bacterial compositions are mixtures of different bacterial isolates obtained from a stable microbial community of an adult organism (poultry or swine) that are introduced into a young organism in order to control colonization by pathogens *(15)*. This approach has proved very effective by establishing a stable gastrointestinal microflora that prevents *Salmonella* colonization through direct competition for attachment sites *(21)* or competition between native flora and *Salmonella* for limited nutrients *(12)*.

The use of bacterial compositions for biocontrol of pathogens on sprouts assumes that the native microbial community from "mature" sprouts (i.e., approximately 1-week-old specimens ready for consumption) would reduce growth of pathogens if inoculated onto seeds. We tested this assumption by using the microbial community from sprouts

* Author for correspondence. Tel: 215-836-3782; Fax: 215-233-6406; E-mail: amatos@arserrc.gov.

† Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

cultured under aseptic conditions in the lab and under typical production conditions in a commercial setting as inoculants, and comparing their ability to control the growth of *Salmonella* with a single strain identified as a successful antagonist during screening performed in our lab. Our experimental approach involved the use of undefined community samples in order to verify that the overall community reduced pathogen growth before subsets of the community (i.e., mixtures of isolates) are tested.

Effective competitive exclusion treatments involve microbial communities that are stable and functionally active. Stability is defined as the degree of change in community response over time. We used community-level physiological profiling (CLPP) to monitor community change over time and assess the functional ability of the microbial communities. Stable and functionally active microbial communities may be better adapted to resist invasion and proliferation of foodborne pathogens by potential mechanisms of biocontrol such as better use of resources or by making fewer resources available to invaders. CLPP was performed to determine whether inoculants resulted in greater community stability and increased functional potential, thereby providing insight into potential mechanisms for biocontrol. CLPP is based on the rapid assessment of multiple physiological responses of microbial communities *(16)*. The original CLPP approach involved the response of communities to 95 separate sole carbon sources as determined by growth and concomitant reduction of a redox sensitive dye within a microtiter plate *(8)*. The resulting phenotypic fingerprint is a valuable tool for assessing temporal or spatial variation in heterotrophic communities and was employed in the present study to evaluate effects of inoculation treatment on the overall community stability during the course of sprout development. The Biolog CLPP approach has limited value for assessing actual differences in the physiological capabilities because of the selective enrichment within the assay caused by high substrate concentrations (>100 mM) and long incubation times (1 to 4 days) *(7, 20)*. A new CLPP approach based on detection of substrate use via an oxygen-sensitive fluorophore reduces incubation time and substrate concentrations, allowing for more functionally relevant data *(9)*. Bacterial density on the sprouts, based on both culture and direct microscopic methods, was assessed to provide baseline information on what fraction of the community can be readily cultured, an important factor in potential definition of bacterial compositions.

MATERIALS AND METHODS

Generation of *Salmonella* cocktail inoculum. Four different serovars of *Salmonella*, resistant to 1,000 μg/ml of streptomycin and 100 μg/ml of nalidixic acid, were used in this study: *Salmonella* Newport H1275, *Salmonella* Anatum F4317, *Salmonella* Stanley H0558, and *Salmonella* Infantis F4319. All these strains were isolated from sprout-related outbreaks and were provided by Dr. Patricia Griffin of the Centers for Disease Control and Prevention (Atlanta, Ga.). The bacteria were maintained in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) amended with 25% glycerol at −80°C. Single colonies from each one of these species were inoculated into 2 ml of TSB and incubated at 37°C with agitation (250 rpm) for 24 h. From these cultures, 100 μl was transferred into 25 ml of fresh TSB and incubated with agitation for an additional 24 h. From these cultures, 10 ml was centrifuged at 5,000 × *g* for 10 min. The pellets were washed twice in 10 ml of sterile 0.1% peptone water. The final cell pellets were taken up in 10 ml of 0.1% peptone water. From these bacterial suspensions, 40 μl for each *Salmonella* species was added to 400 ml of sterile distilled water and 400 g of alfalfa seeds (International Specialty Supply, Cookeville, Tenn., lot number NS9-130) in a sterile stomacher bag with a filter membrane on the side (Stomacher Circulator, model 400, Seward, Norfolk, UK). The seeds were mixed by hand for 3 min. The bacterial inoculum was then drained off and the inoculated alfalfa seeds were placed in a sterile glass tray lined with sterile paper towels under a biological safety cabinet (Forma Scientific, Class IIA/B3, Marietta, Ohio). After 24 h, the inoculated seeds were placed in a sterile glass beaker, covered with sterile aluminum foil paper, and stored at 4°C. The concentration of the inoculum on the seeds was determined by stomaching 25 g of inoculated seeds in 200 ml of 0.1% peptone water for 3 min at medium speed, preparing decimal dilutions in sterile 0.1% peptone water, and plating onto tryptic soy agar (TSA; Difco, Becton Dickinson) plates and TSA plates with streptomycin (1,000 μg/ml) and nalidixic acid (100 μg/ml). Bacterial colonies were enumerated after 48 h of incubation at 37°C.

Screening of potential antagonists. As a first step, the native microflora of various types of sprouts (alfalfa, clover, radish, onion, sunflower, mung beans, broccoli, mustard, and wheat grass) were examined. Several methods for recovering native microflora from sprouts, with particular emphasis on fluorescent pseudomonads and psychrophilic microorganisms, were assessed. Additionally, the sprout-associated microbial communities were characterized by examining microbial richness (e.g., the number of types of bacteria), microbial diversity (e.g., microbial richness and evenness), and CLPP. Various culture-dependent microbial identification technologies, including Vitek and Biolog were used to identify the isolates. Competitive exclusion bioassays were used to select nondeleterious microorganisms native to sprouts as potential antagonists against foodborne pathogens. Also, several well characterized biological control agents were evaluated. Over 700 isolates were tested individually for their ability to exclude *Salmonella* species from growing alfalfa sprouts. Each isolate was maintained in TSB amended with 20% glycerol at −80°C. Single colonies from isolation streaks on TSA plates were inoculated into 2 ml of TSB and incubated at about 24°C with agitation (250 rpm) for 24 h. From this culture, 50 μl were transferred into 2 ml of fresh TSB and incubated for an additional 24 h. The cells were then harvested by centrifugation at 7,000 × *g* for 10 min and washed twice, first in sterile distilled water and then sterile tap water. Cell densities were adjusted to an absorbance of 0.1 at 600 nm (approximately 10^8 CFU/ml) by dilution in sterile tap water. This bacterial suspension was then used as the inoculum to be tested. About 35 *Salmonella*-inoculated alfalfa seeds were then soaked in 2 ml of the inoculum suspension for 2 h. Ten seeds per each of three vials were then aseptically transferred and grown for 7 days.

***Pseudomonas fluorescens* 2-79 inoculum.** This strain was originally isolated from the rhizosphere of wheat and it has been shown to suppress take-all, a major root and crown disease of wheat and barley caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici (22, 23)*. *P. fluorescens* was maintained in TSB amended with 20% glycerol at −80°C. Single colonies from isolation streaks on TSA plates were inoculated into 2 ml of TSB and incubated at about 24°C with agitation (250 rpm) for

24 h. From this culture, 50 μl was transferred into 2 ml of fresh TSB and incubated for an additional 24 h. The cells were then harvested by centrifugation at 7,000 × *g* for 10 min and washed twice, first in sterile distilled water and then sterile tap water. Cell densities were adjusted to an absorbance of 0.1 at 600 nm (approximately 10^8 CFU/ml) by dilution in sterile tap water. This bacterial suspension was then used as the *P. fluorescens* 2-79 inoculum. About 35 *Salmonella*-inoculated alfalfa seeds were then soaked in 2 ml of the inoculum suspension for 2 h. Ten seeds per each of three vials were then aseptically transferred and grown for 7 days.

Market sprouts inoculum. Alfalfa sprouts were obtained from Winn Dixie Supermarkets (Merritt Island, Fla.). Sprouts (1.5 g) were submerged in 15 ml of 100 mM potassium phosphate–0.1% peptone buffer (pH 6.8) in a 50-ml sterile centrifuge tube (Corning Inc., Corning, N.Y.) and shaken vigorously by hand for 30 s. The 50-ml sterile centrifuge tube containing the sprout tissues was then placed in a sonicator water bath (Branson 1510, Branson Ultrasonic Corp., Danbury, Conn.) for 7 min at 20°C. The centrifuge tubes were vortexed for 30 s to disperse the bacteria that were dislodged by sonication. This bacterial suspension was then used as the market sprout inoculum. For enumeration, 0.5 ml of sample from the suspension was serially diluted in 0.85% NaCl and plated on TSA plates. About 35 *Salmonella*-inoculated alfalfa seeds were soaked in 2 ml of the inoculum suspension for 2 h. Ten seeds per each of three vials were then aseptically transferred and grown for 7 days.

Lab sprouts inoculum. Alfalfa seeds were germinated in small, capped, glass vials in the laboratory for 7 days. Ten alfalfa seeds (International Specialty Supply, lot number NS9-130) were placed in each vial. Each vial contained filter membranes soaked with sterile tap water (250 μl per vial), and the vials were placed in an enclosed glass chamber. Sprouts were watered aseptically with 250 μl of sterile tap water on day 5. On day 7, the sprouts were harvested and sprout-associated bacteria recovered using sonication as described above. This bacterial suspension was then used as the lab sprouts inoculum. About 35 *Salmonella*-inoculated alfalfa seeds were soaked in 2 ml of the inoculum suspension for 2 h. Ten seeds per each of three vials were then aseptically transferred and grown for 7 days.

Germination and growth of alfalfa sprouts. *Salmonella*-inoculated alfalfa seeds with added inoculants were germinated and grown in the laboratory for 7 days. Each glass capped vial contained filter membranes soaked with sterile tap water (250 μl per vial) and 10 alfalfa seeds per vial. The vials were placed in an enclosed glass chamber. Temperature and relative humidity were monitored twice daily and maintained at about 24°C and 60%, respectively. Sprouts were watered aseptically with 250 μl of sterile tap water on day 5. The control treatment consisted of alfalfa seeds soaked in sterile tap water, and no single biocontrol agent or sprout-derived microbial suspensions were added.

Recovery and enumeration of bacteria from alfalfa sprout surfaces. On day 1 (24 h after planting), day 3 (72 h after planting), and day 7 (168 h after planting), the sampled sprouts were submerged in 15 ml of 100 mM potassium phosphate–0.1% peptone buffer (pH 6.8) in a 50-ml sterile centrifuge tube (Corning Inc.) and shaken vigorously by hand for 30 s. The 50-ml sterile centrifuge tube containing the sprout tissues was then placed in a sonicator water bath for 7 min at 20°C. The centrifuge tubes were vortexed for 30 s to disperse the bacteria that were dislodged by sonication. For the enumeration of total mesophilic aerobes, a 0.5-ml sample from the suspension was serially diluted in 0.85% NaCl and plated on TSA. Following incubation at 30°C for 48 h, bacterial colonies were enumerated and phenotypically characterized based on colony size, pigmentation, form, elevation, margin, and surface. During each sampling the following data were recorded: number of germinating seeds, seedling fresh weight (grams), and observations on sprout appearance.

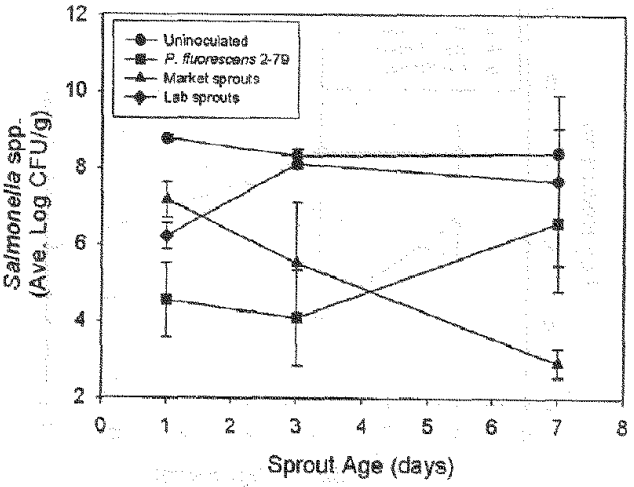

FIGURE 1. *Log reduction in growth of* Salmonella *after introduction of inoculants. Inocula symbols:* ■, P. fluorescens *2-79;* ◆, *lab sprouts, microbial communities derived from laboratory-grown alfalfa sprouts;* ▲, *market sprouts, microbial communities derived from alfalfa sprouts purchased at a local supermarket.*

Calculation of log reduction in the growth of *Salmonella*. The ability of the inoculants to suppress the growth of *Salmonella* on growing alfalfa sprouts was quantified by subtracting the number of *Salmonella* CFU growing on the selective medium, TSA with streptomycin (1,000 μg/ml) and nalidixic acid (100 μg/ml), from the number of *Salmonella* CFU per gram on *Salmonella*-inoculated experimental treatments that received no biocontrol inoculum.

Microbial richness and percentage culturability. Based on colony morphology, the number of different types of bacteria (microbial richness) in each sprout surface sample plated onto TSA without antibiotics was recorded. The different sprout-associated bacterial colonies grown from sprout surface suspensions on TSA plates as described above were isolated and characterized phenotypically by recording colony size, pigmentation, form, elevation, margin, and surface. Percent culturability was determined by dividing the culturable cell density (TSA viable counts) by the total cell density (acridine orange—see below).

Acridine orange staining. The acridine orange staining method was used as previously described by Hobbie *(14)*. One milliliter of each sprout-derived microbial community suspension sample was transferred to a 2-ml microcentrifuge tube, and 0.5 ml of 2% filtered-sterilized formaldehyde solution was added. The fixed samples were stored in the refrigerator at 4°C until analysis.

Live-dead staining. Components A and B (A = Syto9; B = propidium iodide) of the live-dead BacLight bacterial viability kit (Molecular Probes, Eugene, Oreg.) were thawed *(1, 11)*. Three microliters of premixed live-dead stain (2:1 Syto9:propidium iodide) per ml of sample was prepared. Nine hundred microliters of filtered (0.2 μm) sterilized deionized water was added to a 100-μl microbial community suspension sample to obtain a final dilution of 1:10. Three microliters of the live-dead stain mixture per ml of sample in Eppendorf tubes (2 ml) was added and incubated

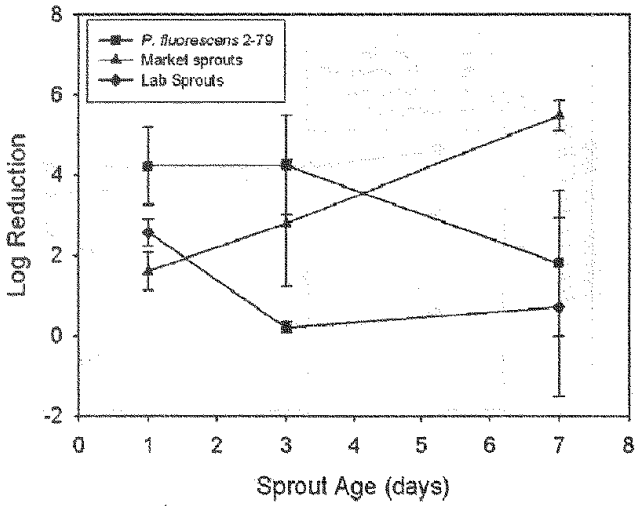

FIGURE 2. *Total microbial counts (acridine orange staining), total culturable counts, and percentage culturability. Inocula symbols: ●, uninoculated;* ■, P. fluorescens *2-79;* ◆, *lab sprouts, microbial communities derived from laboratory-grown alfalfa sprouts;* ▲, *market sprouts, microbial communities derived from alfalfa sprouts purchased at a local supermarket. Filled circles represent treatments with* Salmonella*-inoculated alfalfa seeds. Logarithmic cells per milliliter, logarithmic CFU per milliliter, and percentage culturability values are the averages of three replicate samples. The error bars represent one standard deviation from the average value.*

in the dark at room temperature for 15 min. Samples were vacuum filtered onto 25-mm diameter, 0.2-μm (pore size) black polycarbonate filters (Isopore, Millipore, Billerica, Mass.) with shiny side up. The 10^0 and 10^{-1} live-dead filters were placed side by side on a labeled microscope slide, and a drop of Resolve immersion oil was added before covering the filters with a cover glass. Live-dead slides were enumerated within 4 h of sampling and filtration. Cells were manually counted at ×1,000 magnification using a Zeiss Axioskop 2 microscope with an epifluorescence attachment (Zeiss, Thornwood, N.Y.). The Alpha Vivid filter set (Omega, Inc., Brattleboro, Vt.) (excitation 475 ± 40 nm, emission 510 nm and above) was used as recommended by Molecular Probes for the BacLight kit.

CLPP. Two types of plates were used to conduct CLPP: Biolog GN2 plates and BD oxygen biosensor plates (BD Biosciences, Billerica, Mass.). The Biolog approach involves inoculation of environmental samples (e.g., suspensions of bacteria from sprout surfaces) into 96-well Biolog GN2 plates (Biolog, Hayward, Calif.) and subsequent generation of a multivariate profile of sole carbon source use based on the reduction of a tetrazolium dye as a result of microbial respiration *(7)*. Microbial suspensions from each sprout type were diluted (1:10) in sterile distilled water to reduce exogenous carbon input to the plates and contaminant reduction of dye in the control well, which contains no carbon source before inoculation into the microtiter plates (150 μl per well). The plates were incubated in the dark at 25°C for 96 h, and the optical density (590 nm) of each well was measured automatically every 2 h using a Biotek EL320 plate reader (Biotek, Inc., Winooski, Vt.).

The BD oxygen biosensor system is a detection system in which as oxygen is consumed the biosensor fluoresces, providing a signal that can be directly correlated to use of a specific substrate. The BD oxygen biosensor system is an oxygen-sensitive fluorescent compound (Tris 1,7-diphenyl-1,10 phenanthroline ruthenium [II] chloride) embedded in a gas-permeable and hydrophobic matrix permanently attached to the bottom of a multiwell plate. The concentration of oxygen in the vicinity of the dye is in equilibrium with that in the liquid media. Oxygen quenches the dye in a predictable concentration-dependent manner. The amount of fluorescence correlates directly to the rate of oxygen consumption in the well, which in turn can relate to any sort of reaction that can be linked to oxygen consumption (http://www.bdbiosciences.com). In this study, 11 carbon substrates (300 ppm) were used in BD plates: sucrose, fructose, xylose, rhamnose, alanine, glycine, asparagine, casamino acids, sodium acetate, malic acid, and TSB. On the day of sampling (days 1, 3, and 7), 50 μl of minimal salts was added to all wells within one BD plate. Then 50 μl of each substrate was added into each well in a single column. Finally, 50 μl of microbial suspension from sprouts was inoculated on the BD plates. Plates were read on a Dynex MFX microplate fluorometer (Dynex Technologies, Chantilly, Va.) at 485-nm excitation and 604-nm emission using the top reading mode. Plates were incubated at 30°C, and readings were obtained every 15 min for 24 h.

Statistical analysis. All experiments run with germinating sprouts were performed in a completely randomized design with three replicates. A replicate consisted of sprout tissue sampled from a single glass vial containing 10 seeds. The bacterial counts were all logarithmically transformed prior to statistical analysis. Significant differences among treatments were determined at a significance level of 0.05 by using single factor analysis of variance (ANOVA).

The time to peak fluorescence generated in the BD oxygen biosensor plates was analyzed by ANOVA to determine the effects of inocula on the responses. The analysis was performed for all 11 responses (carbon sources tested). Means separations were performed using the pairwise least significant difference separation method at the $P = 0.05$ significance level.

The pattern of color development from the CLPP generated with Biolog GN2 plates was assessed at an equivalent average well color development to eliminate potential density-dependent bias. The individual well responses for the plate reading at an average well color development of 0.50 absorbance units, corresponding to 40 to 50 h of incubation depending on sample, was used for subsequent multivariate analysis. Principal component analysis using a covariance matrix was employed to evaluate the relative degree of similarity of samples in multidimensional (i.e., 95 carbon source variables) space (SPSS Software, Inc., Chicago, Ill.).

RESULTS

Seed germination and sprout appearance. The average percentage germination of alfalfa seeds at the time of harvest on days 1, 3, and 7 was 24 ± 18%, 61 ± 18%, and 58 ± 14%, respectively. The alfalfa seedlings average fresh weight at the time of harvest on days 1, 3, and 7 was 0.05 ± 0.01, 0.08 ± 0.02, and 0.12 ± 0.04 g, respectively. The sprouts looked healthy, with white, hairy roots and bright green leaves, and seed coats were detached at the time of harvest on day 7. The introduction of inoculants to the alfalfa seeds had no apparent deleterious effects on the growing alfalfa sprouts.

***Salmonella* viable counts on TSA with streptomycin and nalidixic acid.** The number of *Salmonella* on the uninoculated treatment ranged from 8.15 to 8.96 log CFU/g

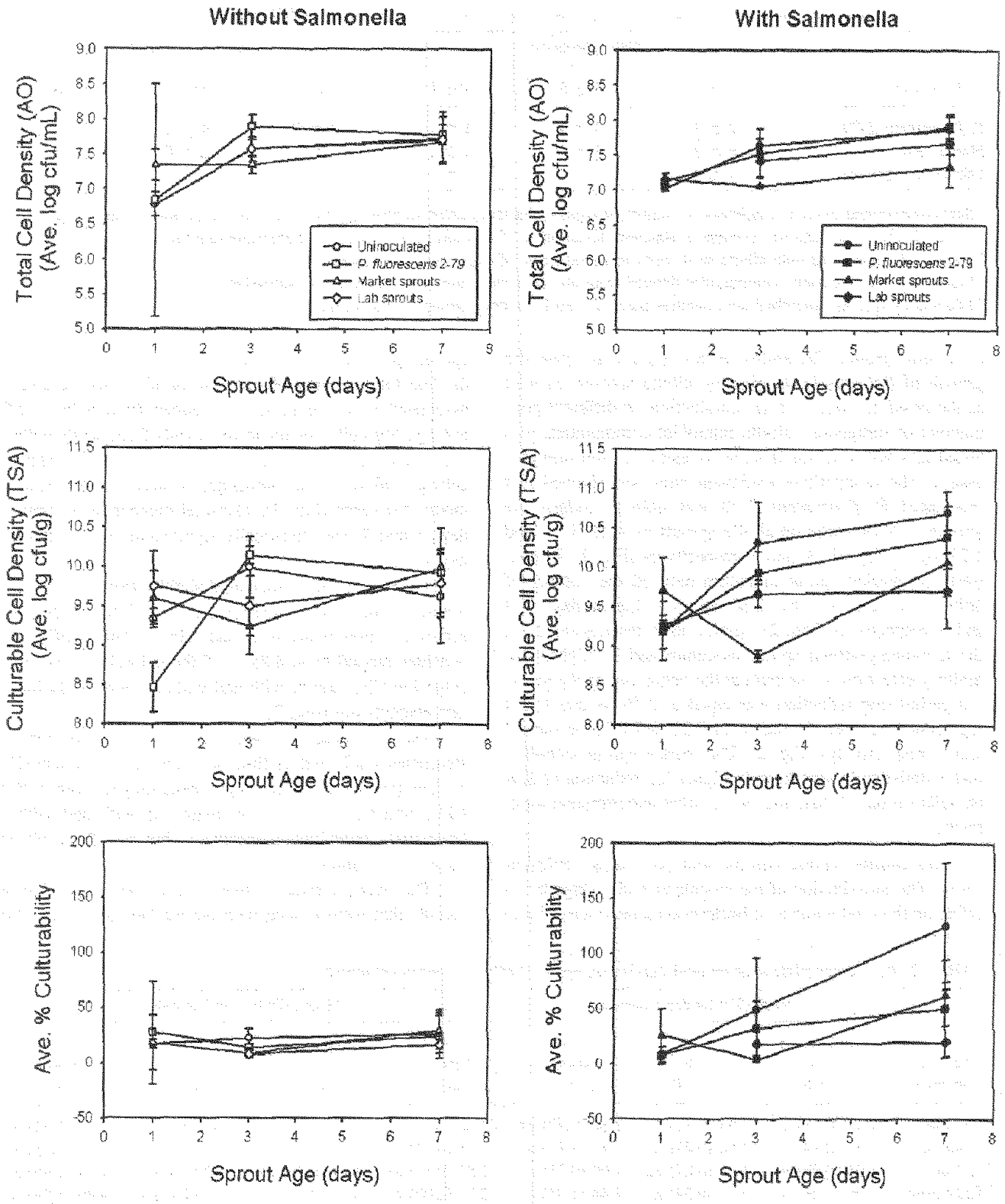

FIGURE 3. Salmonella *cell density (TSA plates with streptomycin (1,000 μg/ml) and nalidixic acid (100 μg/ml). Inocula symbols:* ●, *uninoculated;* ■, P. fluorescens *2-79;* ♦, *lab sprouts;* ▲, *market sprouts. Logarithmic cells per milliliter values are the averages of three replicate samples. The error bars represent one standard deviation from the average value.*

(Fig. 1). The lowest average numbers of *Salmonella* were obtained on day 7 (market sprouts inoculum), 2.91 log CFU/g, and on days 1 and 3 (*P. fluorescens* 2-79), 4.55 and 4.09 average log CFU/g, respectively. These data show a distinct difference between the suppression of *Salmonella* by the market sprout inoculum and the potential biocontrol agent, *P. fluorescens* 2-79. Although *P. fluorescens* 2-79 suppressed the growth of *Salmonella* early on, the market sprouts inoculum had an even greater inhibitory effect during the later growing period of alfalfa sprouts.

Log reduction of *Salmonella* growth on alfalfa. *P. fluorescens* 2-79 is a root colonizer of potential interest for the biological control of foodborne pathogenic *Salmonella*

TABLE 1. *Microbial richness, that is, the number of different types of bacteria growing on TSA plates*[a]

Experimental treatment	With *Salmonella*[b]			Without *Salmonella*		
	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
P. fluorescens 2-79	2 ± 1	3 ± 1	3 ± 1	2 ± 0	4 ± 1	3 ± 0
Market sprouts[c]	6 ± 1	1 ± 0	4 ± 1	7 ± 1	2 ± 0	5 ± 1
Laboratory sprouts[d]	4 ± 1	5 ± 1	4 ± 1	4 ± 1	6 ± 1	5 ± 0

[a] Sprout-associated microbial richness = number of types of bacterial colonies growing on TSA plates at the time of sampling (days 1, 3, and 7 after germination). Average ± standard deviation at 95% confidence level of three replicate samples.
[b] *Salmonella* inoculated onto alfalfa seeds prior to introduction of experimental treatments.
[c] Market sprouts, microbial communities derived from alfalfa sprouts purchased at a local supermarket.
[d] Laboratory sprouts, microbial communities derived from laboratory-grown alfalfa sprouts.

on alfalfa sprouts. The ability of this strain to suppress the growth of *Salmonella* on growing alfalfa sprouts, as well as the possible effects of its introduction on different populations of indigenous alfalfa microbial communities, was tested in vivo using small-scale competitive exclusion bioassays. The competitive exclusion bioassays showed that inoculated *P. fluorescens* 2-79 was able to reduce the growth of *Salmonella* on alfalfa sprouts by 4.22, 4.24, and 1.81 log on days 1, 3, and 7, respectively (Fig. 2). In contrast, the market sprout inoculum reduced the number of *Salmonella* cells by 1.61, 2.80, and 5.48 log on days 1, 3 and 7, respectively (Fig. 2). Similar to *P. fluorescens* 2-79, the laboratory-grown sprout inoculum had its highest inhibitory effect on *Salmonella* at the beginning of the grow-out period (log reduction was equal to 2.56 on day 1, but log reductions then decreased to 0.21 and 0.71 on days 3 and 7, respectively) (Fig. 2). The market sprout inoculum had a statistically significantly higher log reduction of *Salmonella* on day 7 than any of the other experimental treatments.

AO counts, viable counts, and percentage culturability. The introduction of the inoculants had no significant effect on the total number of bacteria associated with alfalfa sprouts germinated from seeds with and without *Salmonella*. The total number of bacteria (acridine orange counts) associated with alfalfa sprouts ranged from 6.78 to 7.90 average log cells per ml in treatments from seeds without *Salmonella* inoculum and from 7.02 to 7.91 average log cells per ml in alfalfa sprouts germinated from *Salmonella*-inoculated seeds (Fig. 3). The total number of bacteria on days 3 and 7 was statistically significantly higher than on day 1.

The viable counts increased slightly from day 1 to day 3 and remain at the same level on day 7 for all four experimental treatments with and without *Salmonella*. Cell densities ranged from 8.88 to 10.69 and 8.46 to 10.14 average log CFU per g, with and without *Salmonella* inoculum respectively (Fig. 3).

The percentage culturability, while variable, was not significantly affected by time or inoculation treatment (Fig. 3). The percentage of culturable cells ranged from 2.94 to 100% and 0.25 to 81% for treatments with and without *Salmonella* inoculum, respectively, but was 2 to 4% for most of the samples.

The average ratio of live to dead cells on 7-day-old sprouts that were treated with the market sprout inoculum

TABLE 2. *BD oxygen plate data on peak heights (normalized relative fluorescent units)*[a]

Carbon substrate	Mean (SD) for day 1 inocula			Mean (SD) for day 7 inocula			
	P. fluorescens 2-79 (n = 6)	Lab (n = 4)	Market (n = 4)	Uninoculated (n = 6)	*P. fluorescens* 2-79 (n = 6)	Lab (n = 4)	Market (n = 4)
Sucrose	1.44 (0.15) B[b]	1.55 (0.32) B	2.60 (0.78) AB	2.06 (0.96) AB	1.59 (0.21) AB	1.94 (0.70) AB	3.64 (2.32) A
Fructose	1.53 (0.14) A	1.90 (0.42) A	2.82 (1.41) A	2.05 (0.41) A	1.64 (0.33) A	1.89 (0.28) A	3.27 (2.15) A
Xylose	1.03 (0.08) B	1.41 (0.07) AB	1.66 (0.31) AB	1.53 (0.11) AB	1.24 (0.10) AB	1.59 (0.37) AB	2.38 (1.46) A
Rhamnose	1.96 (0.26) A	3.12 (0.34) A	4.44 (1.94) A	3.71 (2.00) A	4.10 (2.15) A	3.67 (1.17) A	4.40 (1.86) A
Alanine	2.32 (0.74) CD	2.01 (0.17) D	6.99 (0.33) A	2.19 (0.69) CD	4.32 (2.03) BC	2.12 (0.45) CD	6.20 (0.58) AB
Glycine	1.17 (0.39) B	1.03 (0.07) B	2.12 (0.55) A	1.02 (0.04) B	1.05 (0.08) B	1.05 (0.05) B	1.29 (0.27) B
Asparagine	1.44 (0.25) C	1.90 (0.15) BC	3.41 (0.29) AB	1.75 (0.49) BC	2.30 (0.72) BC	1.90 (0.66) BC	4.32 (1.84) A
Casamino	1.39 (0.10) B	1.41 (0.06) B	3.42 (1.96) A	1.43 (0.13) B	1.63 (0.19) B	1.62 (0.15) B	1.96 (0.31) AB
Na acetate	2.06 (0.57) B	1.93 (0.16) B	7.43 (0.21) A	2.86 (2.29) B	4.48 (1.98) AB	3.20 (1.33) B	6.55 (0.53) A
Malic acid	1.13 (0.05) C	1.31 (0.07) BC	1.67 (0.15) A	1.18 (0.11) BC	1.14 (0.10) BC	1.16 (0.05) BC	1.36 (0.15) B
TSB	1.54 (0.17) B	1.56 (0.22) B	2.91 (0.18) AB	1.52 (0.19) B	1.99 (0.37) AB	3.47 (1.69) A	2.37 (1.31) AB

[a] Data analyzed by ANOVA to determine the effects and interaction of time and inocula on the responses. The analysis was performed for all 11 responses. Mean separations were performed using the Bonferroni least significant difference (LSD) separation method at the $P = 0.05$ significance level.
[b] Means for a given response with no letter in common are significantly different by Bonferroni LSD ($P < 0.05$) mean separation test.

TABLE 3. *BD oxygen plate data on time to peak (days)*[a]

Carbon substrate	Mean (SD) for day 1 inocula			Mean (SD) for day 7 inocula			
	P. fluorescens 2-79 (n = 6)	Lab (n = 4)	Market (n = 4)	Uninoculated (n = 6)	*P. fluorescens* 2-79 (n = 6)	Lab (n = 4)	Market (n = 3)
Sucrose	0.58 (0.12) A[b]	0.57 (0.05) A	0.40 (0.04) BC	0.43 (0.07) ABC	0.50 (0.04) AB	0.53 (0.06) AB	0.32 (0.04) C
Fructose	0.68 (0.13) A	0.66 (0.04) A	0.48 (0.04) AB	0.48 (0.09) AB	0.66 (0.18) A	0.48 (0.07) AB	0.39 (0.04) B
Xylose	0.68 (0.37) A	0.93 (0.05) A	0.65 (0.05) A	0.72 (0.19) A	0.89 (0.12) A	0.63 (0.13) A	0.55 (0.02) A
Rhamnose	0.75 (0.18) A	0.73 (0.07) A	0.48 (0.04) BC	0.61 (0.08) ABC	0.70 (0.06) A	0.62 (0.05) AB	0.39 (0.04) C
Alanine	0.51 (0.23) BC	0.85 (0.04) A	0.41 (0.02) C	0.72 (0.15) AB	0.53 (0.04) BC	0.77 (0.11) AB	0.36 (0.04) C
Glycine	0.04 (0.00) C	0.66 (0.42) AB	0.86 (0.05) A	0.91 (0.23) A	0.30 (0.15) BC	0.67 (0.39) AB	0.80 (0.18) AB
Asparagine	0.55 (0.05) A	0.46 (0.03) AB	0.32 (0.02) B	0.48 (0.16) AB	0.43 (0.08) AB	0.42 (0.08) AB	0.29 (0.04) B
Casamino	0.42 (0.06) A	0.44 (0.02) A	0.28 (0.01) AB	0.37 (0.14) AB	0.38 (0.09) AB	0.33 (0.04) AB	0.22 (0.01) B
Na acetate	0.60 (0.27) ABC	0.82 (0.01) A	0.38 (0.03) BC	0.82 (0.16) A	0.60 (0.07) ABC	0.69 (0.12) AB	0.32 (0.02) C
Malic acid	0.43 (0.07) AB	0.47 (0.02) AB	0.29 (0.02) B	0.57 (0.24) A	0.34 (0.04) AB	0.35 (0.06) AB	0.24 (0.02) B
TSB	0.40 (0.06) AB	0.51 (0.15) A	0.27 (0.02) B	0.34 (0.01) AB	0.38 (0.04) AB	0.40 (0.15) AB	0.23 (0.01) B

[a] Data analyzed by ANOVA to determine the effects and interaction of time and inocula on the responses. The analysis was performed for all 11 responses. Means separations were performed using the Bonferroni least significant difference (LSD) separation method at the $P = 0.05$ significance level.

[b] Means for a given response with no letter in common are significantly different by Bonferroni LSD ($P < 0.05$) mean separation test.

introduced into alfalfa seeds without *Salmonella* was 1.42 ± 0.41. On *Salmonella*-inoculated seeds the average live-to-dead ratio was 1.22 ± 0.47.

Richness. The average number of morphological types of bacterial colonies observed on the TSA plates varied from 2 to 7. No experimental treatment effect was observed (Table 1). The culturable microbial composition of the lab and market sprouts inocula, as well as the microflora associated with the treated sprouts, included a great number of gram-negative rods. Microorganisms identified include *Pseudomonas fulva, Pantoea ananatis, Pseudomonas veronii, Pseudomonas marginalis, Pantoea dispersa, Pantoea agglomerans, Escherichia hermanii, Pseudomonas synxantha, Pseudomonas syringae tabaci* (pv), *Pseudomonas fluorescens* biotype A, *Pseudomonas fluorescens* biotype G, *Pseudomonas asplenii, Erwinia rhapontici, Stenotrophomonas maltophila, Acinetobacter lwoffii, Acinetobacter johnsonii, Enterobacter pyrinus,* and *Pseudomonas putida* biotype A. This a general survey of the type of microorganisms found on sprouts. The level of effort necessary for such a thorough cataloguing of organisms from all those treatments was beyond the scope of the work and was the basis for our use of CLPP to address the experimental objectives.

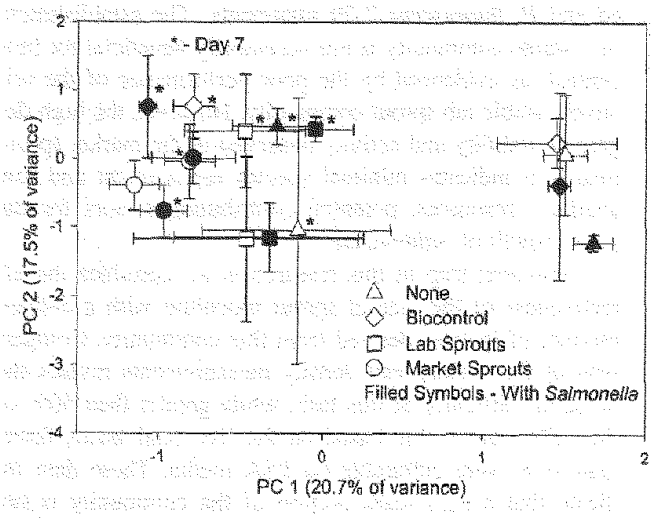

FIGURE 4. *CLPP Biolog. Principal component analysis plot. None, no inoculants introduced onto germinating alfalfa seeds; lab sprouts, microbial communities derived from laboratory-grown alfalfa sprouts; market sprouts, microbial communities derived from alfalfa sprouts purchased at a local supermarket. Values are the averages of three replicate samples. The error bars represent one standard deviation from the average value.*

Community-level physiological profiling using Biolog plates. Principal component 1 clearly separated day 1 and day 7 samples for the untreated and biocontrol treated sprouts, indicating a temporal shift in the physiological profiles within those treatments (Fig. 4). Separation along principal component 2 was less clear, although differences between the day 1 and day 7 samples from the lab community treated are apparent. Market sprout treated samples showed no discernable change with time. There was no consistent effect of *Salmonella* inoculation of seed.

Community-level physiological profiling using BD plates. Microbial communities associated with the market spout treatment displayed a shorter time to peak (i.e., faster response; Tables 2 and 3) toward most of the 11 carbon sources tested (Fig. 5). ANOVA indicated a significant inoculation effect, and least significant difference results reveal the effect is due to a day effect. ANOVA on market sprout communities on peak heights and time to peak showed a statistically significant difference on the response from the market sprouts microbial communities.

DISCUSSION

These results indicate that microbial communities from mature sprouts can serve as effective competitive exclusion

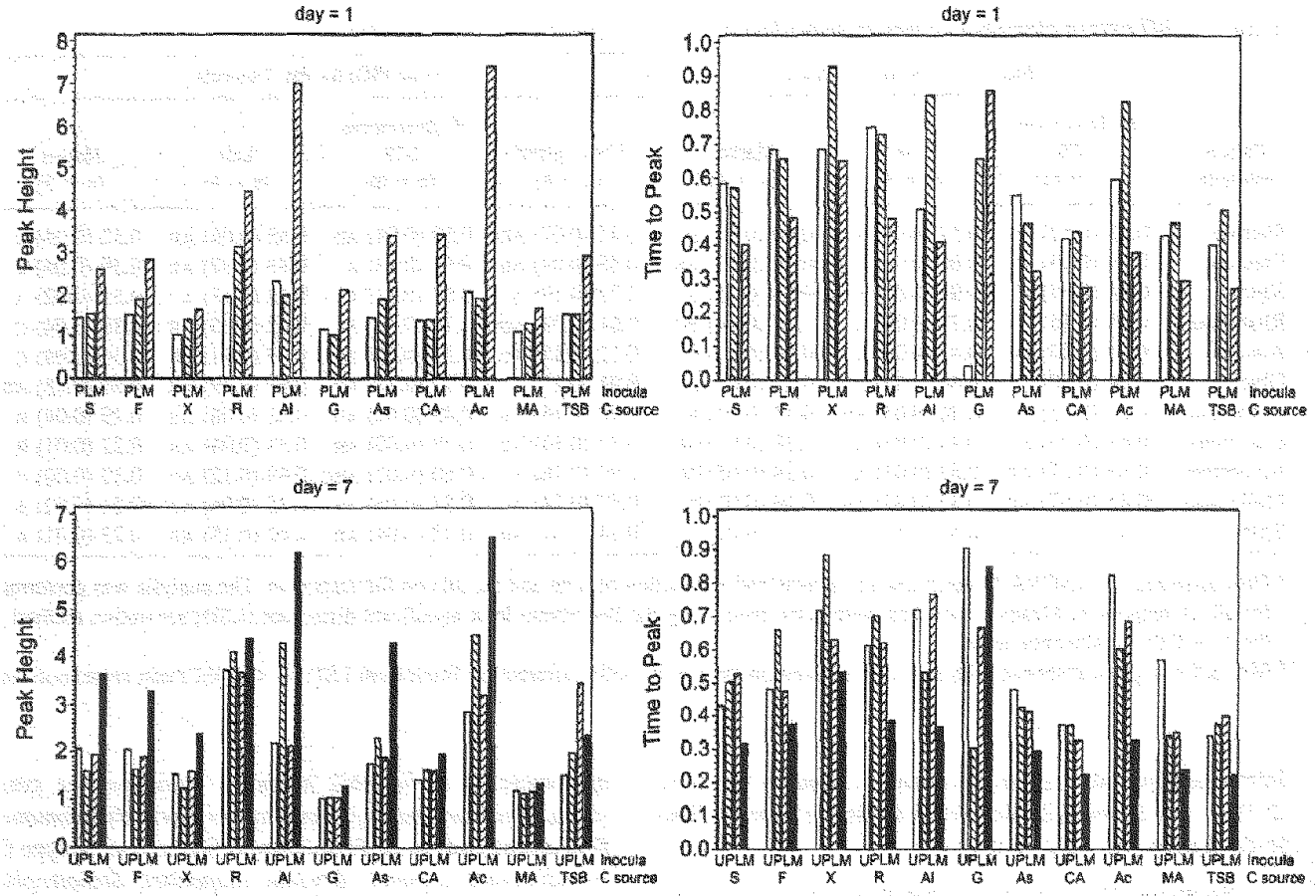

FIGURE 5. *CLPP-BD microtiter plates. Peak heights and time to peak. P,* Pseudomonas fluorescens *2-79; L, lab sprouts, microbial communities derived from laboratory-grown alfalfa sprouts; M, market sprouts, microbial communities derived from alfalfa sprouts purchased at a local supermarket. Carbon substrates used: S, sucrose, F, fructose, X, xylose, R, rhamnose, Al, alanine, G, glycine, As, asparagine, C, casamino acids, S, sodium acetate, M, malic acid, and TSB, tryptic soy broth. Values are the averages of three replicate samples. The error bars represent one standard deviation from the average value.*

inoculants; the market sprout inoculum caused greater log reduction in *Salmonella* than the single strain biocontrol agent (*P. fluorescens* 2-79) after 7 days. Community inoculum generated from seedborne microbes alone (i.e., lab sprout inoculum) did not confer any sustained protection from *Salmonella*, indicating that colonization and selection during commercial production lead to a more robust community with greater antagonistic or competitive community properties or both. Our data clearly indicate that the market sprout community had greater functionality, as evidenced by faster use of an array of substrates, suggesting competition with the *Salmonella* for available resources as a potential mode of action. The increase in log reduction with time in the market sprout treatment further supports the role of competition. The inverse temporal effect of *P. fluorescens* 2-79 on *Salmonella* relative to the market sprout community suggests that the former may act through some as yet undefined antagonistic mechanisms but is unable to compete as effectively over time. Bacterial compositions that include an antagonistic strain with a competitive mixture may be a useful area of future study.

The Biolog CLPP data indicate that inoculation of the seed with the mixed communities, particularly with that obtained from the market sprouts, resulted in the rapid establishment of a stable community. Bacterial numbers rapidly increased in all treatments by day 1, but community composition as indicated from the phenotypic profiles shifted during the course of sprout development in the uninoculated and *P. fluorescens* 2-79 treatments. The establishment of a stable community is not necessarily beneficial for biocontrol, as evidenced by the poor performance of the relatively stable lab sprout community. However, the high degree of stability and activity observed in the market sprout treatment indicates minimal species replacement and low available resources, potential contributing factors for the poor growth of *Salmonella*.

The next step in this research is to reproduce the effectiveness of the market sprout inoculum with a defined mixture of isolates derived from this community. Comparison of the various cell density measurements reveals the potential difficulty of this task; while greater than 50% of the cells were viable based on the live-dead assay, fewer than 10% were culturable on TSA media. These data indicate that a significant portion of the community is not readily cultured, either because of physiological shifts of culturable types to a viable but nonculturable state *(3)* or because of the presence of specific types of organisms that we cannot readily culture. The latter factor would mean that improved culturing approaches may be required to develop an effective bacterial composition.

ACKNOWLEDGMENTS

We would like to thank the following individuals for their valuable technical assistance in collecting data included in this study: Manuel Delgado (InterAmerican University of Puerto Rico, Arecibo, Puerto Rico), NASA Advanced Life Support Microbial Ecology staff (Kennedy Space Center, Fla.), and Kimberly Wall (USDA, Eastern Regional Research Center, Wyndmoor, Pa.).

REFERENCES

1. Boulos, L., M. Prevost, B. Barbeau, J. Callier, and R. Desjardins. 1999. LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J. Microbiol. Methods* 37:77–86.
2. Brennerova, M. V., and D. E. Crowley. 1994. Direct detection of rhizosphere colonizing *Pseudomonas* spp. using an *Escherichia coli* rRNA promoter in a Tn*7-lux* system. *FEMS Microbiol. Ecol.* 14: 319–330.
3. Byrd, J. J., S.-H. Xu, and R. R. Colwell. 1991. Viable but nonculturable bacteria in drinking water. *Appl. Environ. Microbiol.* 57:875–878.
4. Center for Infectious Disease Research and Policy (CIDRAP) News. 3 October 2002. FDA cites mung bean sprouts in renewing warning against eating raw sprouts. Available at: http://www.cidrap.umn.edu/cidrap/content/fs/food-disease/news/beansprout.html.
5. Fett, W. F. 2002. Factors affecting the efficacy of chlorine against *Escherichia coli* O157:H7 and *Salmonella* on alfalfa seed. *Food Microbiol.* 19:135–149.
6. Food and Drug Administration (FDA) Talk Paper. T02-37. 2 October 2002. Available at: http://www.fda.gov/bbs/topics/ANSWERS/2002/ANS01164.html. Accessed 7 October 2004.
7. Garland, J. L., K. L. Cook, C. A. Loader, and B. A. Hungate. 1997. The influence of microbial community structure and function and community-level physiological profiles, p. 171–183. *In* H. Insam and A. Rangger (ed.), Microbial communities: functional versus structural approaches. Springer, Heidelberg.
8. Garland, J. L., and A. L. Mills. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole carbon source utilization. *Appl. Environ. Microbiol.* 57:2351–2359.
9. Garland, J. L., M. S. Roberts, L. H. Levine, and A. L. Mills. 2003. Community-level physiological profiling using an oxygen-sensitive fluorophore in a microtiter plate. *Appl. Environ. Microbiol.* 69:2994–2998.
10. Genovese, K. J., R. C. Anderson, R. B. Harvey, T. R. Callaway, T. L. Poole, T. S. Edrington, P. J. Fedorka-Cray, and D. J. Nisbet. 2003. Competitive exclusion of *Salmonella* form the gut of neonatal and weaned pigs. *J. Food Prot.* 66:1353–1359.
11. Gregori, G., S. Citterio, A. Ghiani, M. Labra, S. Sgorbati, S. Brown, and M. Denis. 2001. Resolution of viable and membrane-compromised bacteria in freshwater and marine waters based on analytical flow cytometry and nucleic acid double staining. *Appl. Environ. Microbiol.* 67:4662–4670.
12. Ha, S. D., S. C. Ricke, D. J. Nisbet, D. E. Corrier, and J. R. DeLoach. 1994. Serine utilization as a potential competition mechanism between *Salmonella typhimurium* and a chicken cecal bacterium. *J. Food Prot.* 57:1074–1079.
13. Health and Human Services News. 9 July 1999. Consumers advised of risks associated with raw sprouts [press release]. Available at: http://www.cfsan.fda.gov/~lrd/hhssprts.html.
14. Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nucleopore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* 33:1225–1228.
15. Lloyd, A. B., R. B. Cumming, and R. D. Kent. 1977. Prevention of *Salmonella typhimurium* infection in poultry by pre-treatment of chickens and poults with intestinal extracts. *Aust. Vet. J.* 53:82–87.
16. Mills, A. L., and J. L. Garland. 2001. Application of physiological profiles to assess community properties, p. 135–146. *In* ASM manual of environmental microbiology. ASM Press, Washington, D.C.
17. Neilands, J. B., and S. A. Leong. 1986. Siderophores in relation to plant disease. *Annu. Rev. Plant Physiol.* 37:187–208.
18. Nisbet, D. J. 2002. Defined competitive exclusion cultures in the prevention of enteropathogen colonization in poultry and swine. *Antonie van Leeuwenhoek* 81:481–486.
19. Raaijmakers, J. M., M. Vvlami, and J. T. de Souza. 2002. Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* 81:537–547.
20. Smalla, K., U. Wachtendorf, H. Heuer, W.-T. Liu, and L. Forney. 1998. Analysis of BIOLOG GN substrate utilization patterns by microbial communities. *Appl. Environ. Microbiol.* 64:1220–1225.
21. Snoeyenbos, G. H., O. M. Weinack, and C. F. Smyser. 1979. Further studies on competitive exclusion for controlling salmonellae in chicks. *Avian Dis.* 23:904–914.
22. Weller, D. M., and R. J. Cook. 1983. Suppression of take-all of wheat by seed treatments with fluorescent pseudomonads. *Phytopathology* 73:463–469.
23. Weller, D. M., B.-X. Zhang, and R. J. Cook. 1985. Application of a rapid screening test for selection of bacteria suppressive to take-all of wheat. *Plant Dis.* 69:710–713.
24. Winthrop, K. L., M. S. Palumbo, J. A. Farrar, J. C. Mohle-Boetani, S. Abbott, M. E. Beatty, G. Inami, and S. B. Werner. 2003. Alfalfa sprouts and *Salmonella kottbus* infection: a multistate outbreak following inadequate seed disinfection with heat and chlorine. *J. Food Prot.* 66:13–17.